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Effect of shigatoxin-1 on arachidonic acid release by human glomerular epithelial cells[1]

Douglas I. Schmid and Donald E. Kohan

Division of Nephrology, University of Utah School of Medicine and Salt Lake Veterans Affairs Medical Center, Salt Lake City, Utah, USA

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Background. Altered arachidonic acid (AA) metabolism has been implicated in the pathogenesis of renal injury in the hemolytic uremic syndrome (HUS). However, there is very little information of the effect of shigatoxin (Stx; the putative mediator of renal damage in HUS) on AA release or metabolism by renal cells. Since recent studies have demonstrated that glomerular epithelial cells (GECs) may be important early targets of Stx, the current study was undertaken to examine the effects of Stx on AA release and metabolism by GECs.

Methods. Cultured human GECs were exposed to Stx1 ± lipopolysaccharide (LPS) for 4 to 48 hours followed by determination of ^{3}H-arachidonate release, thromboxane A_2 (TxA_2) and prostacyclin (PGI_2) production, cyclooxygenase (COX) activity, and Western and Northern analyses for phospholipase A_2 (PLA_2) and COX protein and mRNA levels, respectively.

Results. Stx1 increased arachidonate release by GECs. LPS alone had no such effect, but increased arachidonate release in response to Stx1. Stx1-stimulated arachidonate release correlated with elevations in $cPLA_2$ and $sPLA_2$ protein and $cPLA_2$ mRNA levels. Stx1 also increased both TxA_2 and PGI_2 production by GECs; LPS alone did not alter eicosanoid production, but augmented Stx1 effects. Both Stx1 and LPS stimulated COX activity; however, these effects were not additive. Although there was an accompanying elevation of COX-1 and COX-2 mRNA, Stx1 decreased and LPS did not change COX1 and COX2 protein levels.

Conclusions. Stx1 alone or in conjunction with LPS increases arachidonate release and eicosanoid production by human GECs; this effect correlates with increased PLA_2 protein and mRNA levels. To our knowledge, this is the first study identifying the mechanisms of Stx1-stimulated AA release. These results raise the possibility that arachidonate release and metabolism by GECs, and conceivably other renal cell types, are involved in renal injury in HUS.

[1] **See Editorial by Kaplan, Myers, and Leonard, p. 1199**

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The pathogenesis of renal injury in postdiarrheal hemolytic uremic syndrome (HUS) has been hypothesized to be due, in large part, to shigatoxin (Stx) derived from enteric infection with Stx-producing strains of *Escherichia coli* [1, 2]. Stx binds to galactose-α-1,4, galactose-β-1,4, glucose-ceramide (Gb3), is internalized, and ultimately is cytotoxic, at least in part, by directly inhibiting peptide elongation [3]. Stx may also exert pathologic effects by altering a variety of factors involved in regulation of vascular tone, thrombosis, and inflammation [3]. Among these factors, arachidonic acid (AA) metabolites have been frequently invoked. Elevated urinary thromboxane A_2 (TxA_2), a vasoconstrictor, has been described in patients with HUS [4]. Decreased cellular release of prostacyclin (PGI_2), a vasodilator and antithrombotic factor, has been reported in patients with HUS [5]. In addition, red blood cell [6] and blood vessel [5] AA levels have been reported to be reduced in patients with HUS. Despite these studies, there is very little information on the direct cellular effects of Stx on AA metabolism, particularly in the kidney. Such information would be important since AA potentially could exert a wide variety of pathologic renal effects in HUS. For example, free AA can be incorporated into the cellular membrane, act as a second messenger, or be metabolized by a variety of enzymes such as cyclooxygenase (COX), lipoxygenase, or cytochrome p450 [7–9]. Free AA can alter ion channel conductance and intracellular communication via gap junctions [10–12]; activate protein kinase C and mitogen-activated protein kinase isoforms, resulting in a myriad of cellular effects [13, 14]; modify gene transcription [15–17]; and induce a variety of inflammatory reactions [18–21]. Thus, information on the direct effect of Stx on renal cellular AA production and metabolism would be potentially important.

Several cell types could be involved in the renal response to Stx. Glomerular endothelial cell swelling and detachment are prominent in established HUS renal injury [3], suggesting that these cells are important targets of Stx. This conclusion is confounded, however, by the finding that cultured human glomerular endothelial cells

have low intrinsic sensitivity to shigatoxin-1 (Stx1) and that Stx1 cytotoxicity in these cells may be dependent on inflammatory cytokines [22]. There is also evidence that renal epithelial cells are targets of Stx. For example, human renal cortical tubular epithelial cells express abundant Gb3 [23], while kidneys from patients with HUS in its early stages reveal histologically apparent proximal tubular damage [24]. Furthermore, recent studies have found that cultured human glomerular epithelial cells (GECs) and human proximal tubule cells [25] contain large amounts of Gb3 and are highly sensitive to the cytotoxic effects of Stx1 under basal (noncytokine stimulated) conditions [26]. This raises the possibility that Stx interaction with epithelial cells occurs in the early stages of HUS and that such interaction could evoke cellular responses that promote subsequent renal injury. As stated previously in this article, elaboration of AA by these cells, with the potential to promote cell injury, alter hemodynamics, alter platelet aggregation, and promote inflammation (with elaboration of inflammatory cytokines that might alter Gb3 expression by neighboring cells), could be an important early event. Consequently, the first aim of this study was to examine Stx modulation of AA release and metabolism by renal epithelial cells. Since GECs are located close to glomerular endothelial cells and have the potential to modify their biology, GECs were chosen for these initial studies.

In addition to Stx, lipopolysaccharide (LPS) is likely to be present in the circulation of HUS patients [27, 28]. LPS alone does not cause HUS; however, several studies have demonstrated that LPS augments the cytotoxic effects of Stx1 on a variety of cell types [22, 25, 29]. Hence, the possibility exists that LPS could potentiate the effects of Stx on GEC. LPS can modify enzyme activity within the AA metabolic cascade in some cell types; however, its effects on human GEC arachidonate release and eicosanoid synthesis, either alone or in combination with Stx1, are unknown. Thus, the second aim of this study was to examine the effect of LPS, either alone or with Stx1, on AA release and metabolism by GECs.

METHODS

Reagents

Media M199, penicillin/streptomycin (P/S), gentamicin, L-glutamine, mouse Maloney leukemia virus-reverse transcriptase, Superscript II, Oligo dT, dNTPs, SP6 and T7 RNA transcriptases and Taq DNA polymerase were obtained from GIBCO (Grand Island, NY, USA). Endothelial cell growth supplement (ECGS) was from Intracel (Rockville, MD, USA). Fetal bovine serum (FBS) was from Hyclone Laboratories (Logan, UT, USA). Radioimmunoassay (RIA) kits for thromboxane B_2 and 6-keto-prostaglandin $F_{1\alpha}$, horseradish peroxidase-conjugated donkey anti-rabbit IgG and sheep anti-mouse IgG were from Amersham (Arlington Heights, IL, USA). Dynabeads were from Dynal (Lake Success, NY, USA). Mouse anti-human β-actin and ^{3}H-AA were from ICN Biochemicals (Aurora, OH, USA), and primary antibodies, including sheep anti-human COX-1, rabbit anti-human $cPLA_2$ (cytosolic phospholipase A_2), mouse anti-human $sPLA_2$ (secretory PLA_2), and $sPLA_2$ electrophoretic standard were from Cayman Chemical (Ann Arbor, MI, USA). The COX-2 primary antibody was a kind gift of Dr. Steven Prescott (University of Utah, Salt Lake City, UT, USA). Stx1 was isolated in our laboratory by passing bacterial lysate over a Gb3-coated column as previously described [30]. All other chemicals were obtained from Sigma (St. Louis, MO, USA) unless otherwise indicated.

Epithelial cell cultures

Human GECs were isolated and characterized as previously described [26]. Briefly, cortical tissue was obtained from the normal pole of kidneys removed due to renal cell carcinoma. Glomeruli were obtained by sieving and digested with 1 mg/mL of collagenase (type IV; Sigma) until reduced in size by 50 to 75%. Glomerular remnants were suspended in GEC media [M199 + 20% FBS + 100 μg/mL ECGS + heparin (90 μg/mL) + P/S] and plated onto gelatin-coated 10 cm tissue culture dishes. Cells were trypsinized and passaged when approximately 40% confluent. All cells were studied after no more than four passages. Cultures were maintained at 37°C in a 5% CO_2 environment.

Immunofluorescent staining was performed using primary anti-human antibodies, including mouse anti-factor VIII-related antigen (Boehringer Mannheim, Indianapolis, IN, USA), mouse anti-PECAM-1, mouse anti-E-selectin and mouse anti-vimentin (R&D Systems, Minneapolis, MN, USA), mouse anti-cytokeratin 18, mouse anti-cytokeratin 19, mouse anti-pan cytokeratin (reacts poorly with cytokeratin 18 and not with 19; all from Sigma), rabbit anti-WT-1 (Santa Cruz, Santa Cruz, CA, USA), and mouse anti-myosin (Accurate, Westbury, NY, USA). Secondary antibodies were mouse anti-rabbit IgG (Sigma) and rabbit anti-mouse IgG (Boehringer Mannheim). Primary cultures of human umbilical vein endothelial cells (HUVECs; kindly provided by Dr. Tom McIntyre, University of Utah, Salt Lake City, UT, USA), human mesangial cells (HMCs), and human proximal tubular cells (HPT; Clonetics, San Diego, CA, USA) were used as controls for immunofluorescent staining. Freshly isolated human glomeruli also served as controls.

Confluent GEC cultures were growth arrested in M199 + 5% FBS + P/S (growth-arrest media) except for experiments measuring TxA_2 or PGI_2; these were growth arrested in M199 + 100 μg/mL ECGS + 90 μg/mL heparin + P/S (FBS interferes with the RIA). Cells were growth arrested 24 hours before initiating experimental conditions.

Determination of arachidonic acid release and metabolism

For estimating PLA_2 activity, GECs were incubated with 0.5 μCi/mL ^{3}H-arachidonate in experimental media (M199 + 5% FBS + 10 μmol/L indomethacin + 100 U/mL P/S) for one hour. Stx1 ± LPS in experimental media was then added for 4, 24, or 48 hours. The cells were washed and M199 containing 1% fatty-acid free bovine serum albumin (BSA) and 10 μmol/L indomethacin was added for 30 minutes, followed by determination of ^{3}H-arachidonate released into the culture medium using a beta counter (Beckman Coulter, Fullerton, CA, USA). Radioactivity was also measured in a small aliquot of protein to measure total cellular ^{3}H-arachidonate.

Glomerular epithelial cells were treated with Stx1 (10 pg/mL to 10 ng/mL) ± LPS (1 μg/mL) or LPS alone for 4, 24, or 48 hours. Media were assayed by RIA, according to the manufacturer's directions, for TxB_2 or 6-keto-prostaglandin $F_{1\alpha}$, the stable metabolites of TxA_2 and PGI_2, respectively. Total protein as measured by the Bradford method was used to normalize the data [31].

For cyclooxygenase activity determination, growth-arrest media containing Stx1 ± LPS or LPS alone was added to the cells for 4, 24, or 48 hours. Media were then removed, and cells were rinsed with M199. M199 + 0.1% BSA containing a saturating (determined in preliminary experiments) concentration of unlabeled arachidonate (50 μmol/L) was added to the cells for 30 minutes, at which time media were removed and TxB_2 was assayed by RIA.

Western blot analysis

Glomerular epithelial cells were treated with Stx1 ± LPS for 4, 24, or 48 hours. As production of all enzyme isoforms to be studied has been reported to be stimulated in HUVECs by LPS, LPS (1 μg/mL)-treated HUVECs served as positive controls. Cells were lysed and centrifuged for five minutes at 14,000 × *g*, and the supernatant was retained and diluted to a protein concentration of 1 mg/mL. Sample loading buffer was added to the protein mixture. Samples were denatured by heating at 90°C for five minutes, placed on ice, and either loaded onto polyacrylamide gels or stored at −70°C.

Electrophoresis was done using a minigel apparatus (Mini-Protean II; Bio-Rad, Hercules, CA, USA). Samples (30 μg) were electrophoresed on a 5% stacking gel [29:1 acrylamide:bis-acrylamide solution, 125 mmol/L Tris-HCl, pH 6.8, 0.1% sodium dodecyl sulfate (SDS), 0.1% ammonium persulfate, 1 μL/mL tetramethylethylenediamine (TEMED)], separated on a 10% resolving gel (29:1 acrylamide:bis-acrylamide solution, 375 mmol/L Tris-HCl, pH 8.8, 0.1% SDS, 0.1% ammonium persulfate, 0.5 μl/mL TEMED) and transferred onto Hybond-ECL immobilon membranes. Blots were blocked overnight at 4°C in TBST (20 mmol/L Tris-HCl, pH 7.6, 137 mmol/L NaCl, 0.1% Tween-20) + 5% nonfat dry milk + 5% donkey serum + 1% sheep serum. The primary antibody in blocking buffer was added to blots for one hour. Blots were rinsed in blocking buffer, and the horseradish peroxidase-linked secondary antibody (in TBST + 5% donkey serum + 1% sheep serum) was added for one hour. Membranes were rinsed and bands detected by chemiluminescence (Santa Cruz Biochemicals). Autoradiography was performed, and densitometry of the acquired images was measured with an Eagle Eye II gel documentation system and analyzed using Scanalytics software (Stratagene, La Jolla, CA, USA).

Cloning and riboprobe synthesis

A plasmid containing an insert corresponding to the 5′-untranslated region of COX-2 mRNA was a kind gift of Dr. Chris Maloney (University of Utah). All other plasmids were prepared as follows: Oligo dT primed, first-strand complementary DNA (cDNA) was prepared from total cellular RNA isolated from LPS-treated HUVEC using Superscript II reverse transcriptase. Polymerase chain reaction (PCR) was performed on RNA from LPS-stimulated HUVECs using the following primers: $cPLA_2$ (486 bp product), forward, 5′-GCC CTT TAC CTC TTT TCA CCT G-3′, reverse, 5′-CGA CGT CCT TCT CTG GTA TTG-3′; COX-1 (521 bp product), forward, 5′-GTG CTG GAT GGA GAA ATG TAC-3′, reverse, 5′-GGT GTT GAA CAA GAA CTG CTC G-3′; and GAPDH (586 bp product), forward, 5′-GTG AAG GTC GGT GTC AAC GGA TTT-3′, reverse, 5′-CAC AGT CTT CTG AGT GGC AGT GAT-3′.

Purified (Magic PCR Prep; Promega, Madison, WI, USA) and sequenced PCR products were then cloned into a pGEM-T vector (Promega) and subjected to dideoxynucleotide sequence analysis. JM109 cells were transfected with the plasmids and an antisense RNA probe (riboprobe) synthesized using a RiboProbe In Vitro Transcription System (Promega) to a specific activity of 10^9 cpm/μg.

Northern blot analysis

Total RNA was isolated using guanidine thiocyanate-phenol-chloroform extraction. Total RNA (20 μg) was loaded on a 0.9% agarose–0.66 mol/L formaldehyde gel, electrophoresed, transferred to Hybond N membranes, and ultraviolet cross-linked. Membranes were prehybridized with 50% formamide, 5 × standard saline citrate (SSC), 5 × Denhardt's solution, 1% SDS, and 1 μg/mL denatured salmon sperm DNA for four hours at 60°C. The riboprobe was added to the hybridization buffer for 72 hours at 60°C. Each membrane was probed using one specific riboprobe at a time. GAPDH was used to control for RNA integrity and to normalize the data, and the GAPDH riboprobe was only added after densitometry

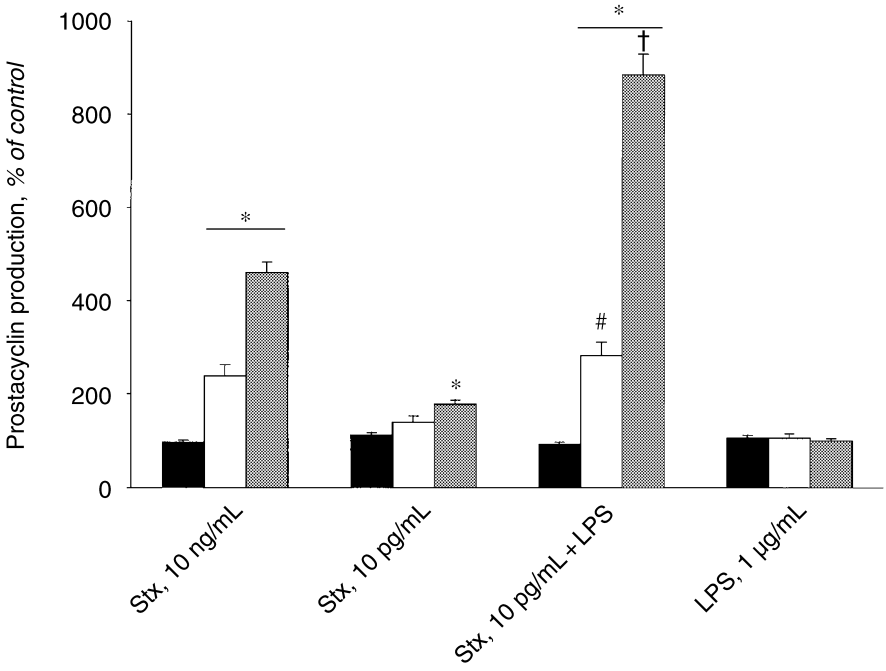

Fig. 1. Effect of shigatoxin-1 (Stx1) and lipopolysaccharide (LPS) on prostacyclin production by glomerular epithelial cells ($N = 6$ for each data point). $*P < 0.01$ vs. controls; $\#P < 0.05$ vs. Stx1 10 pg/mL at same time point; $\dagger P < 0.01$ vs. Stx1 10 pg/mL at same time point. Bar indicates significance at time points covered. Control levels of prostacyclin were 17.7 ± 1.1 pg/mg total cell protein. Symbols are: (■) 4 hours; (□) 24 hours; (▩) 48 hours.

was done on the primary product. No membranes were subjected to stripping. Blots were rinsed with 0.1 × SSC + 0.1% SDS at temperatures up to 80°C as needed to remove nonspecific binding. Densitometry was performed using a Bio-Rad phosphorimaging system and Quantity One software. Conventional autoradiography was also performed.

Statistics

Data are presented as mean values (SEM) with N referring to the number of samples measured for each data point. All data were analyzed using one-way analysis of variance (ANOVA, $\alpha = 0.05$) and evaluated by the Student t test after Bonferroni correction. Values were taken to be significant if $P < 0.05$.

RESULTS

Effect of Stx1 ± LPS on eicosanoid production by GEC

To estimate the effects of Stx1 ± LPS on the AA cascade, we measured the production of COX metabolites, PGI_2, and TxB_2. Stx1 concentrations and exposure times were based on previous studies and were selected so that there was either no (10 pg/mL for up to 24 hours [26]) or minimal (10 ng/mL for 24 hours causes 15% GEC death [26]) cytotoxicity. Stx1 increased GEC biosynthesis of PGI_2 in a time- and dose-dependent manner, with the effect first being evident at 24 hours (Fig. 1). LPS alone did not alter PGI_2 production but significantly augmented the effects of Stx1. TxA_2 levels were also increased after 24 hours of exposure to Stx1 (Fig. 2). This increase was also time- and dose-dependent in a manner similar to that seen with PGI_2 production. LPS alone did not alter production of TxA_2; however, LPS augmented Stx1 effects on TxA_2 production. Lower concentrations of Stx1 (100 fg/mL to 1 pg/mL) did not stimulate eicosanoid release by GEC (data not shown).

Effect of Stx1 ± LPS on PLA_2 activity

Increases in TxA_2 and PGI_2 occurred at similar times and to similar degrees, suggesting that Stx1 increases production of these prostanoids by up-regulating enzymatic activity at a point common to both TxA_2 and PGI_2 biosynthetic pathways. The effect of Stx1 ± LPS on PLA_2 activity, a measure of arachidonate release, was then assessed. Stx1 at 10 ng/mL was used since this concentration clearly stimulated TxA_2 and PGI_2 release by GECs.

Shigatoxin-1 increased arachidonate release by GECs, with a minimal effect noted at four hours and a progressive increase up to 48 hours (Fig. 3). LPS alone did not affect arachidonate release, but significantly augmented the Stx1 response at all time points. To determine whether elevated arachidonate release reflected an increase in PLA_2 enzyme abundance and/or mRNA levels, Western and Northern analyses were performed. Two major PLA_2 isoforms, $cPLA_2$ and $sPLA_2$, were evaluated. With regards to $cPLA_2$, a predominant band at 70 kD was apparent on Western analysis (Fig. 4A). Densitometry of the 70 kD band revealed an increase in $cPLA_2$ protein in response to Stx1 at 48 hours (Fig. 5A). LPS alone slightly increased $cPLA_2$ protein levels at 24 hours and

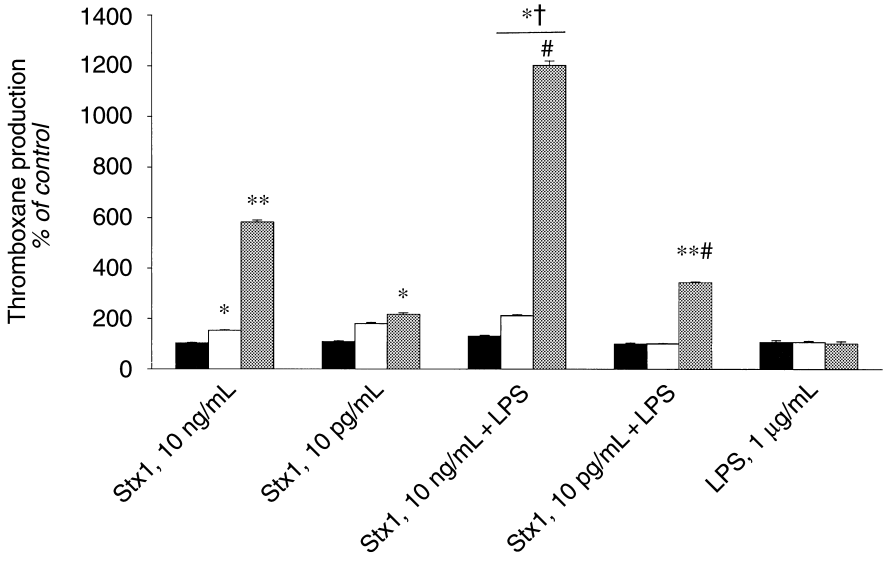

Fig. 2. Effect of shigatoxin-1 (Stx1) and lipopolysaccharide (LPS) on thromboxane A_2 (TxA_2) production by glomerular epithelial cells ($N = 6$ for each data point). $*P < 0.05$ vs. controls; $**P < 0.05$ vs. controls; $\dagger P < 0.01$ vs. Stx1 alone (10 ng/mL) at the same time points; $\#P < 0.01$ vs. LPS alone. Control levels of thromboxane were 716 ± 20 pg/mg total cell protein. Symbols are: (■) 4 hours; (□) 24 hours; (▩) 48 hours.

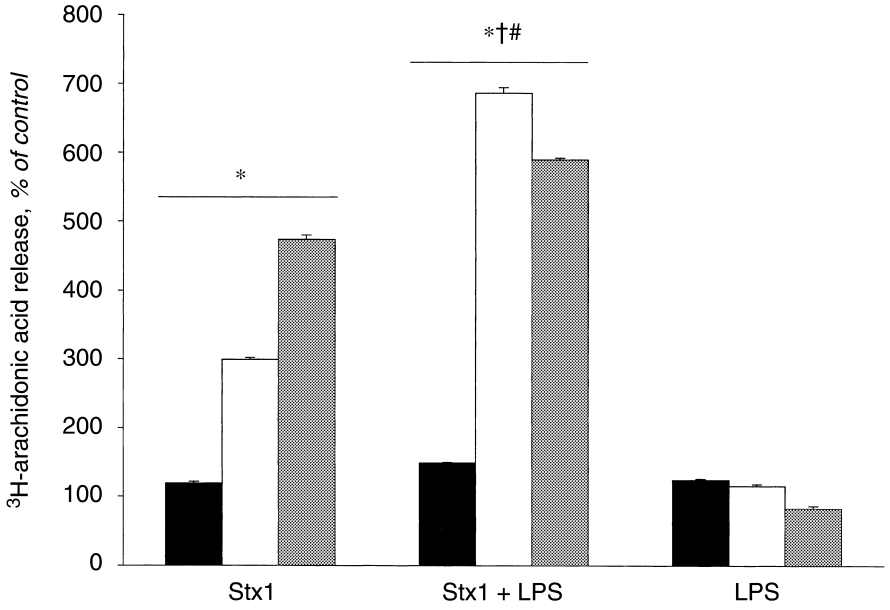

Fig. 3. Effect of shigatoxin-1 (Stx1) (10 ng/mL) and lipopolysaccharide (LPS) (1 µg/mL) on arachidonate release by glomerular epithelial cells ($N = 12$ for each data point). $*P < 0.05$ vs. controls; $\dagger P < 0.05$ vs. Stx1; $\#P < 0.05$ vs. LPS alone. Symbols are: (■) 4 hours; (□) 24 hours; (▩) 48 hours. Bars indicate significance for all time points covered by the bar.

augmented Stx1 action at 24 to 48 hours. $cPLA_2$ mRNA migrated at 3.7 kb on a denaturing gel (Fig. 4B); densitometry on this band revealed that Stx1 increased $cPLA_2$ mRNA levels at 12 and 24 hours (Fig. 5B). LPS also increased $cPLA_2$ levels (24 hours); however, LPS did not augment Stx1 action on $cPLA_2$ mRNA.

The other PLA_2 isoform examined, $sPLA_2$, migrated at 48 and 75 kD on SDS-polyacrylamide gel electrophoresis (SDS-PAGE; Fig. 4A). A purified 14 kD $sPLA_2$ electrophoretic standard also migrated at 48 kD. The 75 kD band may be due to $sPLA_2$ multimers forming in frozen protein samples that are stored in SDS and dithiothreitol (DTT) [32]. Notably, $sPLA_2$ blots performed on fresh samples revealed only a 48 kD band (data not shown). Consequently, the optical density of both bands was used in determination of $sPLA_2$ protein levels. Stx1 increased $sPLA_2$ protein at 24 hours (Fig. 6). Notably, the increase occurred in both the 48 kD and 75 kD bands (data not shown). LPS increased $sPLA_2$ protein at 4 and 24 hours, but had a minimal effect on Stx1-stimulated $sPLA_2$ protein levels.

The $sPLA_2$ antibody for Western blotting was raised against the type II PLA_2 isoforms [33], which have been strongly implicated in the PLA_2 inflammatory response. Despite this, reverse transcription-polymerase chain reaction (RT-PCR) using multiple primer sets directed

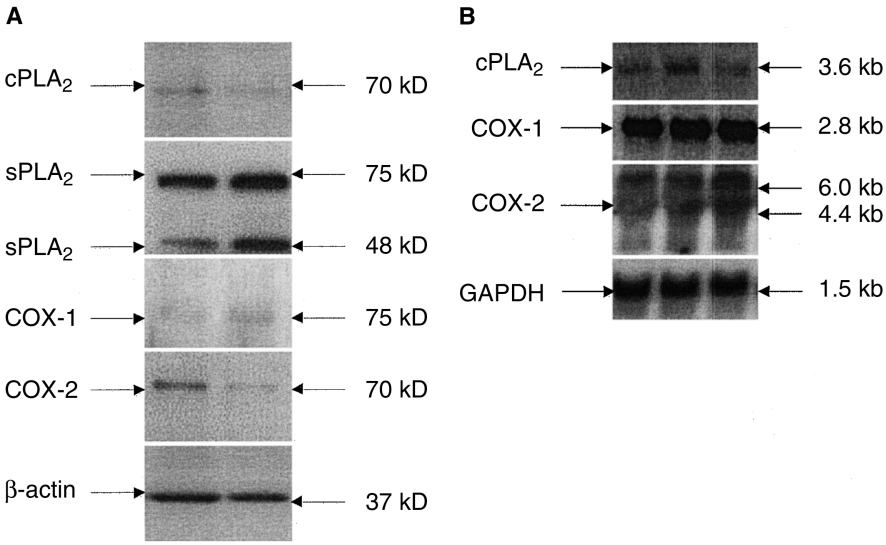

Fig. 4. Western (*A*) and Northern (*B*) blots of PLA_2 and COX isoforms in GECs. Blots are shown from samples treated for 24 hours with Stx1 (10 ng/mL). Abbreviations are: $cPLA_2$, cytosolic phospholipase A_2; $sPLA_2$, secretory phospholipase A_2; COX-1 and -2 cyclooxygenase; and GAPDH, glyceraldeyhde-3-phosphate dehydrogenase. Lanes shown are duplicate (A) or triplicate (B) measurements.

toward type IIA PLA_2 mRNA yielded no products in either LPS-stimulated HUVECs or Stx1-treated GECs (data not shown). Primers were also synthesized for other recently characterized PLA_2 isoforms (type IID, V, and X PLA_2) that were derived from known sequences (GeneBank) and from published reports [34–39]. None of the primers directed at type IID, V, or X yielded PCR products.

Effect of Stx1 ± LPS on COX activity in GECs

The other enzyme family common to PGI_2 and TxA_2 synthesis is the COXs. Having demonstrated increased eicosanoid production and arachidonate release, the Stx1 ± LPS effects on overall COX activity were assessed. COX activity increased in response to Stx1 in a time-dependent manner (Fig. 7). Although LPS increased COX activity by itself, the effect was gone by 48 hours, and it did not augment Stx1 action. To determine whether elevated COX activity reflected an increase in enzyme abundance and/or mRNA levels, Western and Northern blot analyses were performed on the two COX isoforms, COX-1 and COX-2.

Cyclooxygenase-1 protein migrated at 75 kD on SDS-PAGE (Fig. 4A). Stx1 decreased COX-1 protein levels in a time-dependent manner, while LPS had no effect (Fig. 8A). In contrast, Stx1 markedly increased COX-1 mRNA levels (migrated at 2.8 kb; Fig. 4B) at both 12 and 24 hours (Fig. 8B). LPS alone also increased COX-1 mRNA levels at 12 hours (Fig. 8B); however, LPS reduced Stx1-augmented COX-1 mRNA levels.

Cyclooxygenase-2 protein migrated at approximately 70 kD on SDS-PAGE (Fig. 4A). Densitometry on this band indicated that Stx1 decreased COX-2 protein levels while LPS had no effect (Fig. 9A). Stx1 did not significantly alter COX-2 mRNA levels, while LPS had a stimulatory effect (Fig. 9B). In addition, LPS increased COX-2 mRNA levels in conjunction with Stx1; however, this effect was less than that seen with LPS alone.

DISCUSSION

Arachidonic acid can exert a multitude of effects on a variety of cell types [8, 9, 15, 18, 20, 21, 40]. Because of these effects, together with the potentially deleterious effects of AA metabolites [3, 4, 34] and because GECs are exquisitely sensitive to Stx1 [26, 41], we examined whether Stx1 directly stimulates release of AA from GECs. This study reports that Stx1 stimulates free arachidonate release from GECs. Such Stx1-enhanced arachidonate release may, in and of itself, be injurious. Unesterified AA can modify ion channel conductances and intracellular communication via gap junctions [11, 12, 42]. AA can activate protein kinase C and mitogen-activated protein kinase isoforms [13, 14], induce apoptosis [43, 44] and modify gene transcription, most notably that of heat shock proteins [15–17]. AA can also stimulate chemotaxis, superoxide anion formation, degranulation, and adhesion molecule expression in neutrophils [18–21, 45–47], suggesting an interaction between glomerular cells and neutrophils that potentially could result in renal injury. Although unesterified AA is likely to be metabolized quickly or reincorporated into membrane phospholipids, the effective AA concentration within the glomerular microenvironment could reach levels capable of triggering these effects.

Our study also reports that Stx1 increases both TxA_2 and PGI_2 production by cultured human GECs. The significance of this observation in terms of its implications for renal damage in HUS is uncertain. TxA_2 and PGI_2 generally have opposing effects on vascular tone

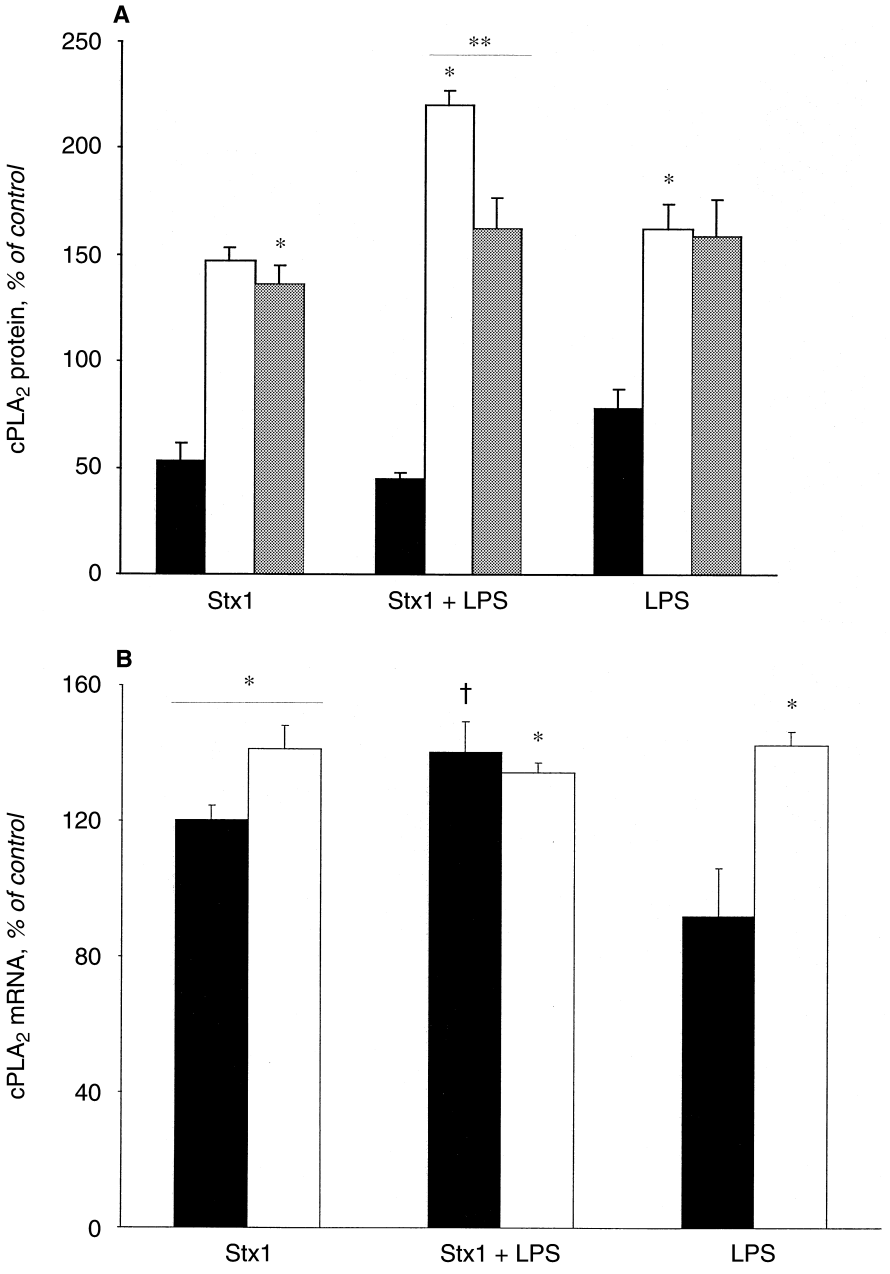

Fig. 5. Effect of shigatoxin-1 (Stx1) (10 ng/mL) and lipopolysaccharide (LPS) (1 μg/mL) on cytosolic phospholipase A_2 ($cPLA_2$) protein and mRNA levels in glomerular epithelial cells. (*A*) Densitometry of $cPLA_2$ Western blot data ($N = 4$ for each data point). Symbols are: (■) 4 hours; (□) 24 hours; (▩) 48 hours. (*B*) Densitometry of $cPLA_2$ Northern analysis ($N = 3$ for each data point). Symbols are: (■) 12 hours; (□) 24 hours. *$P < 0.05$ vs. control; **$P < 0.05$ vs. Stx1 alone at same time point; †$P < 0.05$ vs. LPS at same time point.

and thrombosis [48]. As noted previously in this article, free AA may be deleterious per se and increased eicosanoid production may be of relatively modest biologic significance. Such speculations must be tempered, however, by concerns over extrapolating in vitro patterns of AA metabolism to in vivo conditions. In addition, AA released by Stx could be metabolized by the p450 pathway into a variety of inflammatory and vasoconstrictive substances. While such detailed investigation of the precise metabolic fate of Stx1-stimulated AA in GECs was beyond the scope of the current study, it is important to emphasize that there are a plethora of AA metabolites that have the potential to affect renal biology in HUS. Hence, the major conclusion that can be drawn from the current study is that Stx1, by virtue of stimulating free arachidonate accumulation in GECs, may have the potential to significantly impact renal function in the setting of HUS.

The Stx1-stimulated arachidonate release was associated with increased $sPLA_2$ and $cPLA_2$ protein content at 24 and 48 hours, respectively, suggesting that this effect is due, at least in part, to increased PLA_2 activity and protein levels. Furthermore, Stx1 increased $cPLA_2$ mRNA over a time course similar to that observed with induction of $cPLA_2$ protein, suggesting that increased $cPLA_2$ protein levels reflect enhanced gene transcription

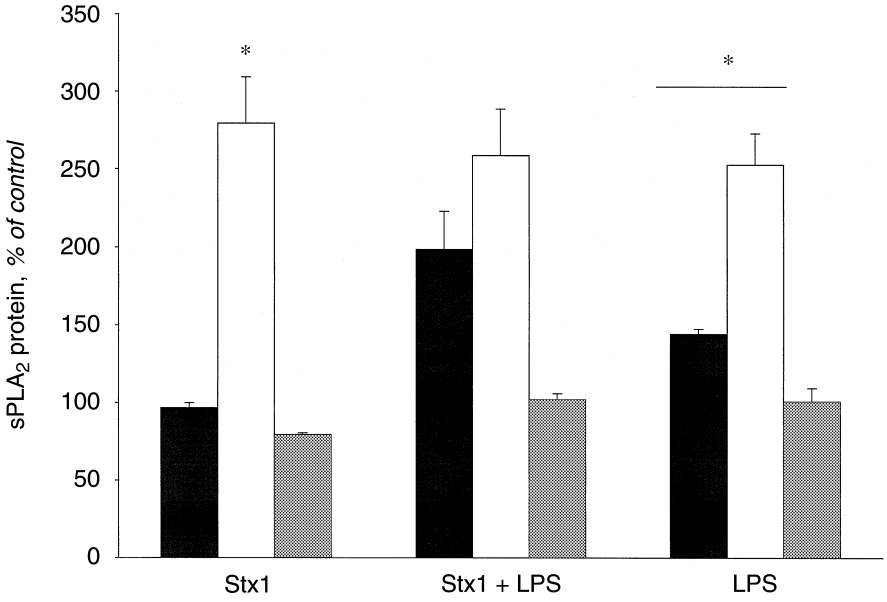

Fig. 6. Effect of shigatoxin-1 (Stx1) (10 ng/mL) and lipopolysaccharide (LPS) (1 μg/mL) effect on secretory phospholipase A_2 ($sPLA_2$) protein levels in glomerular epithelial cells. Data shown are from densitometry of $sPLA_2$ Western blot data ($N = 4$ for each data point). Symbols are: (■) 4 hours; (□) 24 hours; (▩) 48 hours. *$P < 0.05$ vs. controls.

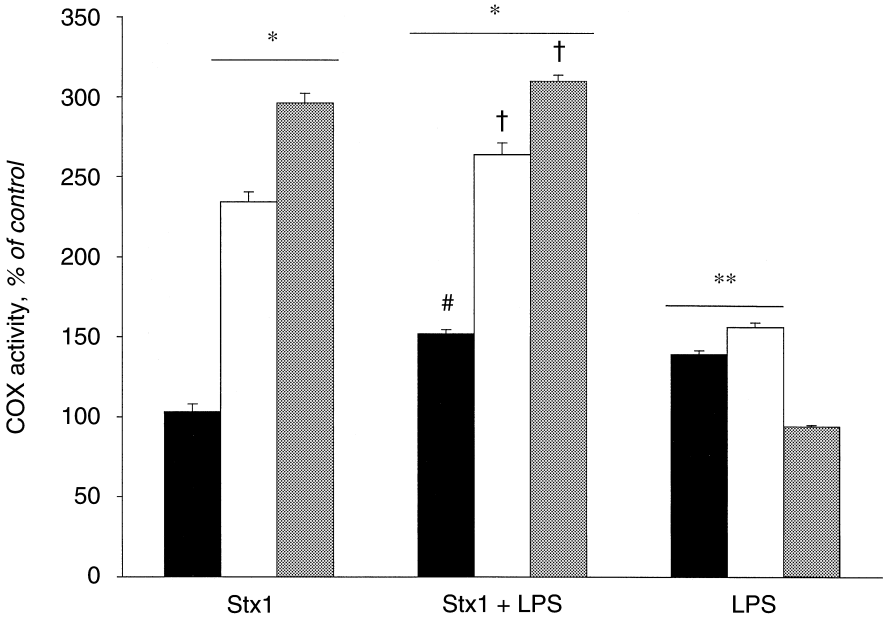

Fig. 7. Effect of shigatoxin-1 (Stx1) (10 ng/mL) and lipopolysaccharide (LPS) (1 μg/mL) on COX activity in glomerular epithelial cells ($N = 6$ for each data point). Symbols are: (■) 4 hours; (□) 24 hours; (▩) 48 hours. *$P < 0.01$ vs. controls; **$P < 0.05$ vs. controls; #$P < 0.05$ vs. Stx1 alone at same time point; †$P < 0.05$ vs. LPS alone at same time point.

with resultant increased mRNA translation. We were unable to detect type II PLA_2 mRNA in GEC under any conditions and using multiple primer sets. This was surprising since the antibody utilized for Western analysis was raised against type II PLA_2 [33]. In addition, no type IID, IB, V, or X PLA_2 mRNA could be detected in GECs by RT-PCR. Hence, it is not possible at this point to be certain as to the role of $sPLA_2$ in Stx1-stimulated arachidonate release in GECs. Nonetheless, it is clear that Stx1 markedly stimulates arachidonate release from GECs, that this effect is most evident after 24 to 48 hours of exposure to the toxin and that enhanced protein synthesis is most likely involved.

Shigatoxin-1 also increased COX activity in GECs; however, this effect was associated with substantial decreases in total COX-1 and COX-2 protein content. Since the amount of active COX protein was not directly assessed, it is unknown whether Stx1 increases the amount of active [49], while decreasing the total, COX protein or whether another, as yet unidentified protein with COX activity, is involved. Proteins with COX activity other than COX-1 and COX-2 have not, to our knowledge,

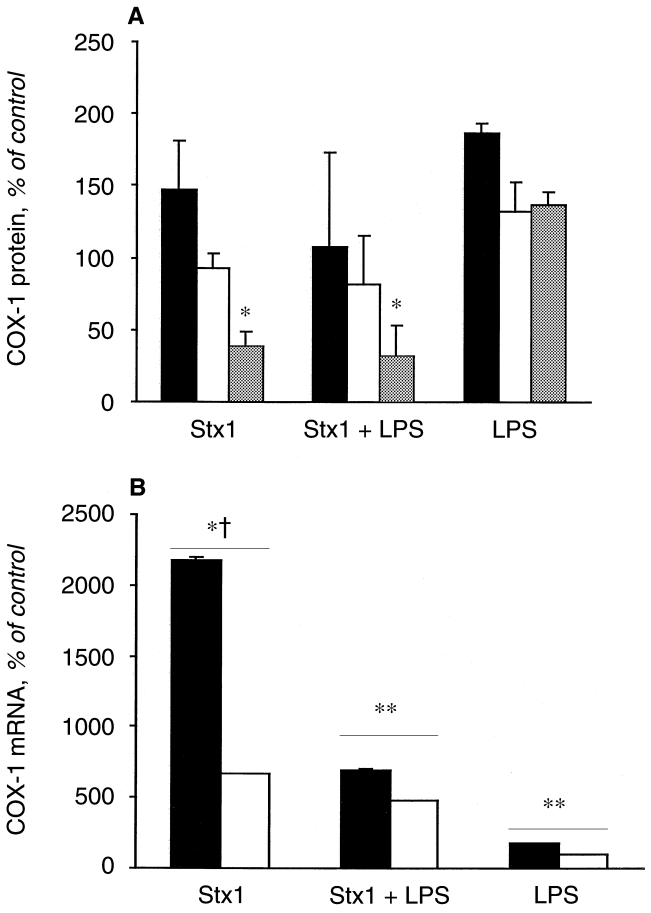

Fig. 8. Effect of shigatoxin-1 (Stx1) (10 ng/mL) and lipopolysaccharide (LPS) (1 μg/mL) on COX-1 protein and mRNA levels in glomerular epithelial cells. (*A*) Densitometry of COX-1 Western blot data (N = 4 for each data point). Symbols are: (■) 4 hours; (□) 24 hours; (▩) 48 hours. (*B*) Densitometry of COX-1 Northern analysis. Symbols are: (■) 12 hours; (□) 24 hours. *$P < 0.01$ vs. controls; **$P < 0.05$ vs. controls; †$P < 0.05$ vs. LPS.

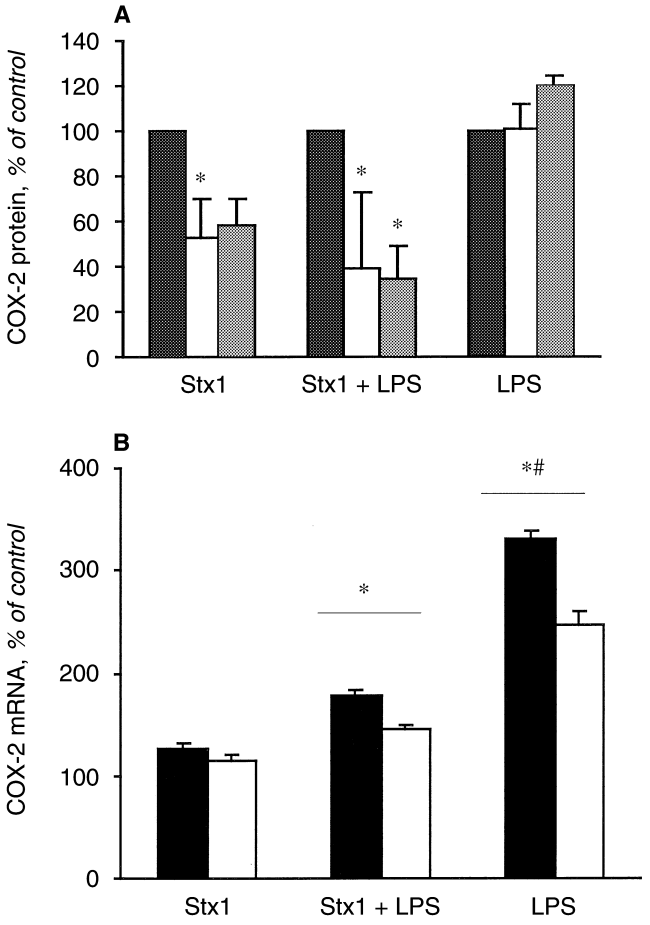

Fig. 9. Effect of shigatoxin-1 (Stx1) (10 ng/mL) and lipopolysaccharide (LPS) (1 μg/mL) on COX-2 protein and mRNA levels in glomerular epithelial cells. (*A*) Densitometry of Western blot data (N = 4 for each data point). Symbols are: (■) 4 hours; (□) 24 hours; (▩) 48 hours. (*B*) Densitometry of COX-2 Northern blot analysis (N = 3 for each data point). Symbols are: (■) 12 hours; (□) 24 hours. *$P < 0.05$ vs. control; #$P < 0.05$ vs. Stx1 + LPS.

been identified. Stx1 also augmented COX-1, but not COX-2, mRNA levels in GECs. The fall in COX-1 protein in the face of increased COX-1 mRNA suggests that Stx1 inhibits translation of COX-1 mRNA and/or accelerates existing COX-1 protein degradation. Examination of these various possibilities, however, was beyond the scope of the current study.

The current study also investigated the effects of LPS on GEC arachidonate metabolism since the endotoxin is likely to be present in the circulation of patients with HUS [27, 28]. Although LPS has been shown to activate the AA metabolic cascade [50, 51], LPS alone did not stimulate production of TxA_2 and PGI_2 by GECs. LPS did have a very modest stimulatory effect on COX activity that was associated with increased COX-1 and COX-2 mRNA levels, an effect that has been noted in other cell types [51–54]. In addition, the endotoxin increased $cPLA_2$ and $sPLA_2$ protein levels to a comparable degree as that seen with Stx1 alone; however, LPS did not enhance arachidonate release. This indicates that Stx1 stimulation of arachidonate release is not merely a reflection of PLA_2 protein levels, but also must relate to enzyme activation.

Lipopolysaccharide did enhance the stimulatory effect of Stx1 on TxA_2 and PGI_2 by GECs. This effect was associated with increases in both arachidonate release and $cPLA_2$ protein levels. LPS and Stx1 together had a smaller stimulatory effect on COX-1 mRNA accumulation as compared to Stx1 alone and also had a smaller stimulatory effect on COX-2 mRNA accumulation as compared with LPS alone; the reasons for these differences are unknown.

In conclusion, our study demonstrates that Stx1 stimulates arachidonate release by GECs and that this effect is associated with increased PLA_2 activity, protein, and mRNA levels. LPS alone had no effect alone, but aug-

ments Stx-stimulated arachidonate release by GECs. In addition, Stx stimulates both TxA_2 and PGI_2 by GECs; this effect is augmented by LPS. These findings raise the possibility that GEC-derived arachidonate and subsequent activation of the AA metabolic cascade play a role in the glomerular pathology observed in HUS.

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Reprint requests to Donald E. Kohan, M.D., Ph.D., Division of Nephrology, University of Utah Health Sciences Center, 50 North Medical Drive, Salt Lake City, Utah 84132, USA.
E-mail: donald.kohan@hsc.utah.edu

APPENDIX

Abbreviations used in this article are: AA, arachidonic acid; BSA, bovine serum albumin; cDNA, complementary DNA; COX, cyclooxygenase; $cPLA_2$, cytosolic phospholipase A_2; DTT, dithiothreitol; ECGS, endothelial cell growth supplement; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Gb3, galactose-α-1,4, galactose-β-1,4 glucose ceramide; GEC, glomerular epithelial cells; HMC, human mesangial cells; HPT, human proximal tubular cells; HUS, hemolytic uremic syndrome; HUVEC, human umbilical endothelial cells; LPS, lipopolysaccharide; M199, Medium 199; PGI_2, prostacyclin; PLA_2, phospholipase A_2; P/S, penicillin/streptomycin; RIA, radioimmunoassay; RT-PCR, reverse transcription-polymerase chain reaction; SDS, sodium dodecyl sulfate; $sPLA_2$, secretory phospholipase A_2; SSC, standard sodium citrate; Stx, shigatoxin; TEMED, tetramethylethylenediamine; TxA_2, thromboxane A_2.

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